Analysis of Antibody Hybridization & Autofluorescence in Touch Samples by Flow Cytometry: Implications for Front End Separation of Trace Mixture Evidence

M. Katherine Philpott, JD/MS, Cristina E. Stanciu, BS, Ye Jin Kwon, MS, Eduardo E. Bustamante, BS, Susan Greenspoon, PhD, Christopher J. Ehrhardt, PhD*

Virginia Commonwealth University, Department of Forensic Science, 1015 Floyd Avenue, Rm 2015, Richmond, VA 23284

Abstract

The goal of this study was to survey optical and biochemical variation in epithelial cell populations deposited onto surfaces through touch or contact and identify specific features that may be used to differentially label and then sort cell populations from separate contributors in a "touch" biological mixture. Previous research has shown that flow cytometry/FACS coupled to fluorescently labeled antibody probes targeting surface antigens such as Human Leukocyte Antigen (HLA) complex can be effective strategy to isolate individual cell populations – and thus generate single source STR profiles – from mixtures composed of blood, buccal, sperm, and/or vaginal cells. However, these methods have not been investigated on contact mixtures, one of the most prevalent forms of biological mixture evidence.

Cell characterizations initially focused on two different protein systems, Human Leukocyte Antigen (HLA) Complex and Cytokeratin (CK) filaments. Hybridization experiments using pan and allele-specific HLA antibody probes showed that surface antigens in controlled touch samples were largely unreactive, in contrast to earlier experiments showing non-specific HLA probe uptake by buccal cells. Cells were also hybridized with cytokeratin probe AE1, which targets CK filament structures 10, 14, 15, 16 and 19. Fluorescence profiles of AE1 hybridized cells were observed to vary slightly across donors, although these differences were not consistent across all sampling days.

We then investigated variations in red autofluorescence profiles (650-670nm) as a potential signature for distinguishing contributor cell populations. Distinct differences in autofluorescence profiles were observed between many pairs of contributors with a median fluorescence varying between ~200 RFU and ~2000 RFU. The variation observed from cell populations from the same individual sampled on different days suggests that a combination of endogenous and exogenous factors may contribute to the cellular autofluorescence signature for a particular individual. To test whether these observed optical differences could potentially be used as the basis for a cell separation workflow, a controlled two person touch mixture was separated into two fractions via Fluorescence Activated Cell Sorting (FACS) using gating criteria based on intensity of 650-670nm emissions, and then subjected to DNA analysis. STR typing of the cell fractions provided partial profiles that were consistent with separation of individual contributors from the mixture.

Current research suggests that cellular autofluorescence signatures can be influenced by the presence of exogenous substances deposited with cells onto a surface through contact. Although the individual compound(s) responsible for the autofluorescence have not been identified they appear associated with a variety of substances including plant material, laboratory gloves, and certain types of ink.

Sample Collection

- Donors either rubbed or held sterile polypropylene conical tube for five minutes.
- Tube swabbed, then cells eluted into solution by manually stirring followed by vortexing for 15 secs in 10 mL of ultrapure water.
- Solution passed through 100 µm filter mesh prior to imaging, Ab hybridization, and/or flow cytometry.
- For two-person mixture studies, cells eluted into 2mL of sterile water. An 860 uL aliquot of each donor's touch cell solution was combined to create a 1:1 mixture (by vol.) for flow analysis, gating, and sorting via FACS. Another 200 uL from each donor was combined to create a mixture that proceeded directly to DNA analysis without sorting.

Antibody Hybridization Analysis

- Aliquots (3mL) of donors' touch cell solutions centrifuged at 5,000xg for five mins.
- Incubated 10 mins with 1 µL of Human Fc Receptor block to increase specificity of antibody binding.
- Cells incubated for 30 mins with either mouse anti-human mAb HLA-ABC-FITC, or anti-acidic cytokeratin probe AE1 followed by secondary Ab anti-mouse IgG1-APC
- Cells washed and resuspended in 1x FACS buffer.
- Flow analysis performed on BD FACSCanto™ II Analyzer

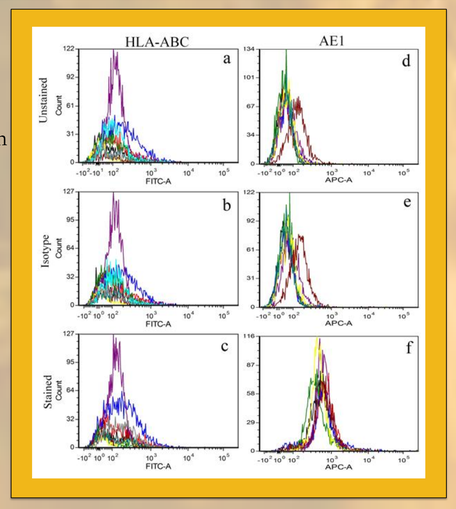


Figure 1. *Hybridization of touch samples with HLA and CK probes. Touch samples failed to uptake the HLA probe (panels a-c). In contrast, all touch samples exhibited uptake of CK probe AE1 when compared with unstained/isotype samples (panels d-f).*

Autofluorescence Analysis

- Analyses performed on Canto and FACSAria flow cytometers
- Cell events falling into the 'large cell' gate based on FSC/SSC analyzed for red autofluorescence (650-670nm)

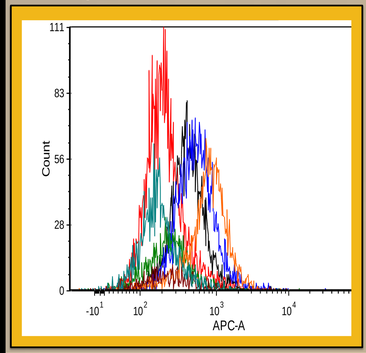
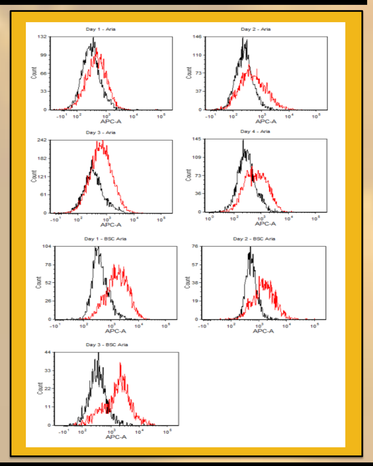


Figure 2 (left). *Overlaid red fluorescence (650-670nm) histograms for cell populations from touch samples deposited by seven individuals.*

Figure 3 (right). *Overlaid red fluorescence histograms for two contributors, D02 (black) and E15 (red), across seven independent sampling days.*



Influence of Exogenous Substances on Autofluorescence

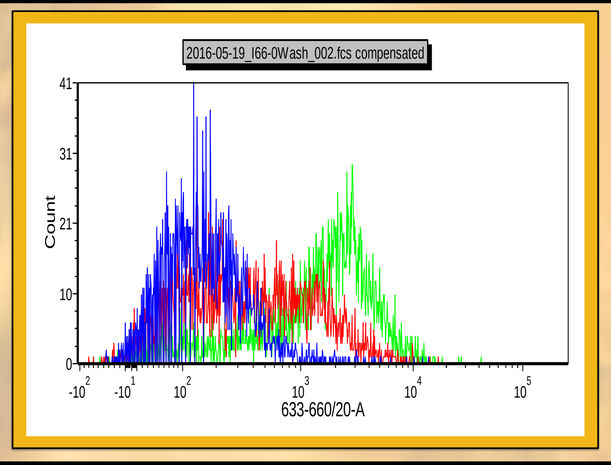
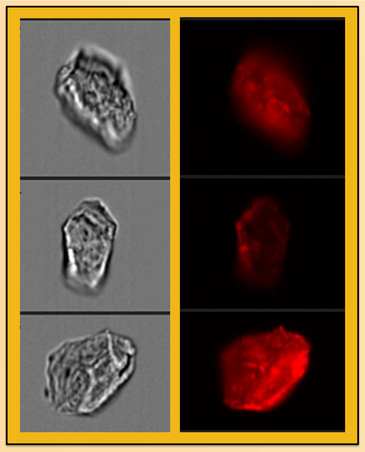


Figure 4 (left). *Overlaid red fluorescence histograms for cell populations from touch samples deposited by a single individual (1) after holding kale (green); (2) after holding kale and subsequent hand washing (red); and (3) without having held kale (blue). One or more fluorescent compounds in the kale (e.g. chlorophyll) appear to have been transferred to cells which were in turn transferred via touch. Although hand washing reduced the number of fluorescent cells, a number of fluorescent cells persisted. Similar results have been observed with other materials such as nitrile gloves and Sharpie markers.*

Figure 5 (right). *Examples of fluorescent cells, post kale handling. Brightfield images are displayed in the left panel; associated images from APC (red) channel are on the right (images captured via Amnis® Imagestream X Mark II).*



Cell Sorting Based on Red Autofluorescence

- Cell events falling into the 'large cell' gate based on FSC/SSC were analyzed for red autofluorescence (650-670nm)
- Analysis and sorting performed on BD FACSAria™ IIU flow cytometer (channel settings: FSC, 200V; SSC, 475V; APC, 400V).

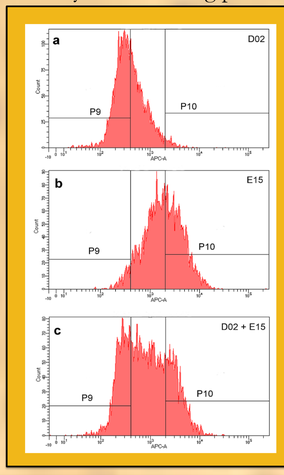
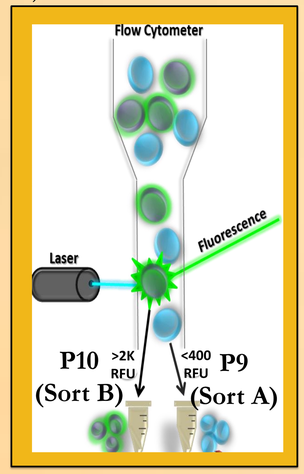


Figure 6. *Sorting gates used for FACS based on APC channel autofluorescence (x-axis: RFUs; y-axis: cell count at given RFU). Histogram profiles for single source samples (panels a, b) were used to define two sorting gates – P9 and P10 – designed for enrichment of one donor relative to the other. Panel c shows the sorting gates plotted against the histogram profile of the two-person cell mixture prior to sorting.*

By designing the sorting gates in this study with an eye toward producing single source profiles, we sacrificed maximal cell recovery for purity of the sort. About half of each donor's cells went unsorted in the region between the two gates. By moving the gates closer together (e.g. 10^3 RFU marks max boundary for P9 and min boundary for P10), more cells could be sorted, and hence available for downstream DNA typing.

Figure 7. *Utilizing FACS, each cell in the mixture sample is passed single file in a fluid stream through a laser beam. Light is scattered by the cell dependent upon its size and granularity, and light of specific wavelengths interacts with intra/extracellular compounds, producing fluorescence. At the same time that sensors are collecting these characteristics, a computer determines whether they satisfy a pre-defined gate (e.g. Fig. 6) and on this basis cells are diverted toward one container or another. For the sort that ultimately produced the profiles displayed in Table 1, 15,406 events/cells were captured in the less fluorescent 'Sort A' (P9) and 10,607 events in the more fluorescent 'Sort B' (P10).*



DNA Typing of Sorted Fractions

- Samples lysed and purified using the DNA IQ System following VA-DFS standard protocols.
- DNA extracts quantitated using the Plexor HY System kit, and amplified via the PowerPlex® Fusion System kit. Capillary electrophoresis conducted on ABI 3500 xL Genetic Analyzer.
- Analytical thresholds set at 88 RFU for fluorescein, 74 RFU for JOE, 114 RFU for TMR-ET, and 80 RFU for CXR-ET. The stochastic threshold was set at 396 RFU

	D3S1358	D1S1656	D2S441	D10S1248	D13S317	Penta E	D16S539	D18S51
D02 Ref	15	12,17.3	14,15	15,16	11,14	10,18	11,13	13,14
E15 Ref	15,17	13,16.3	10,14	13,14	9,11	16,21	10,12	13,18
Pre-sort	15,17	12,13, 16.3	11.3,14	-	-	-	10,11,12,13, 14	13,18,20
Sort A	15	12	-	-	-	-	13	13
Sort B	15,17	-	-	-	13	-	10	-

	D2S1338	CSF1PO	Penta D	TH01	vWA	D21S11	D7S820	D5S818	TPOX
D02 Ref	18,20	11,12	11,15	9	18,19	28,33.2	8,12	12,13	8
E15 Ref	19,25	10,11	11,13	7	16,17	30,31.2	12	11,12	8,12
Pre-sort	17,18,20,25	10	-	7,9	17,18	-	12	-	8
Sort A	24	-	-	-	-	-	-	-	-
Sort B	25	-	-	7	16	-	-	-	-

	DYS391	D8S1179	D12S391	D19S433	FGA	D22S1045
D02 Ref	-	12,13	19,21	14,15	24,25	15,16
E15 Ref	11	14,16	18,20	14,16.2	25,27	15
Pre-sort	-	8,12,13,14,16	18,19,20,21	14,15	23,25,27	16
Sort A	-	12,13	19	14,15	-	-
Sort B	-	-	-	14	-	-

Table 1. *Powerplex fusion profiles developed from donors D02 and E15 reference samples (buccal), unsorted mixture of cells deposited by D02 and E15, and sorted fractions. All alleles detected in Sort A were consistent with donor D02 with the exception of a single 24 allele at locus D2S1338, which did not originate from donor E15 and is likely a drop-in allele. Likewise, all alleles detected in Sort B were consistent with donor E15 with the exception of a single 13 allele at locus D13S317, which did not originate from donor D02 and is likely a drop-in allele. The high degree of dropout and possible drop-in alleles observed are consistent with the extremely low level of template DNA detected in each cell fraction (<50pg).*

Conclusions

- Based on these experiments, HLA probes may not be a useful means of discriminating between touch contributors. And, while contact epithelial cells did uptake AE-1 probe, the degree of variation between individuals may be too limited to design an effective sorting gate. It is possible that probes for individual cytokeratin proteins (vs. pan probes like AE-1), some of which are known to vary with factors such as age, may hold more promise, and is currently being investigated.
- Levels of red autofluorescence varied between individuals and between sampling days. We are currently researching the source and extent of this phenomenon. Preliminary results suggest that skin contact with exogenous fluorescent substances (e.g. plant material, containing chlorophyll) can transfer fluorescent compounds to an individual's cells.
- Gates designed based on red autofluorescence were successfully used to separate a two person mixture into separate fractions prior to extraction; however, the low levels of DNA in the sorted fractions appears to have led to a large degree of allelic drop out and limited degree of allelic drop in. We are working on modified protocols, particularly targeting the sample collection and gating steps to maximize cell (and thus DNA) yield.

Acknowledgements

We would like to thank Dr. Daniel Conrad and Julie Farnsworth at the Flow Cytometry Research Core at the Massey Cancer Center, VCU for providing technical assistance with the project.

This project was funded by the National Institute of Justice Award number 2013-DN-BX-K033 (PI: Ehrhardt)